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Structure and Biological Activity of Gonadotropin-Releasing Hormone Isoforms Isolated from Rat and Hamster Brains

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Abstract
Rat and hamster brain tissues were used to investigate the possible existence of a follicle stimulating hormone (FSH)-releasing factor with similar characteristics to the lamprey gonadotropin-releasing hormone III (lGnRH-III) form proposed in previous reports. The present studies involved isolation and purification of the molecule by high-performance liquid chromatography (HPLC), identification by radioimmunoassay, sequence analysis by automated Edman degradation, mass spectrometry and examination of biological activity. Hypothalamic extracts from both species contained an HPLC fraction that was immunoreactive to GnRH and coeluted with lGnRH-III and 9-hydroxyproline mGnRH ([Hyp^9]GnRH). Determination of primary structure from purified total brain material demonstrated that the isolated molecule was [Hyp^9]GnRH. This is the first report showing the presence of the posttranslationally modified form already known as [Hyp^9]GnRH by primary sequence analysis. The biological activity of distinct GnRH peptides was also tested in vitro for gonadotropin release using rat pituitary primary cell cultures. The results showed that [Hyp^9]GnRH stimulated both luteinizing hormone and FSH release, as already reported, whereas lGnRH-III had no action on the secretion of either gonadotropin.

Mammalian gonadotropin-releasing hormone (mGnRH) is a decapeptide that regulates the reproductive system by stimulating the release of gonadotropin hormones from the anterior pituitary gland [1]. To date, 13 GnRH forms have been characterized in vertebrates and named for the species in which they were initially isolated [2, 3]. All the variants are decapeptides, in which the amino-terminus (pGlu), carboxy-terminus (NH_2) and po-

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sitions 1, 4, 9 and 10 are conserved. Also, another form of GnRH has been reported that is identical to mGnRH except that one amino acid is modified after translation. This form was originally examined in rats and frogs using high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), mass spectrometry and amino acid composition. The structure was deduced to be 9-hydroxyproline mGnRH ([Hyp^9]GnRH) [4].

In mammals 3 of the 13 forms of GnRH have been identified along with [Hyp^9]GnRH. The first form is mGnRH or GnRH-I, which is present in all mammals. The second form is GnRH-II, also known as chicken GnRH-II (cGnRH-II or [His^5,Trp^7,Tyr^8]GnRH). The second form is coexpressed with mGnRH in almost all mammalian species studied to date [5–12], including monkeys and human beings [5, 13–15]. The third molecular form of GnRH is named guinea pig GnRH (gpGnRH) or [Tyr^2,Val^7]GnRH because it was isolated from guinea pig, *Cavia porcellus* [16]. mGnRH and cGnRH-II are thought to be present in all mammals, but gpGnRH has a very limited distribution to date.

Other forms of GnRH have been reported for mammals, but not proven with structural identification. HPLC analysis of brain extracts followed by RIA showed the expression of an unknown GnRH peptide together with mGnRH and cGnRH-II in several mammals [11, 17, 18]. In addition, there are chromatographic and immunological data suggesting the presence of a GnRH-like molecule proposed to be lamprey GnRH-III (lGnRH-III, [His^5,Asp^6,Trp^7,Lys^8]GnRH) in sheep and rat hypothalamic extracts [19, 20]. The last report supports the hypothesis that this lGnRH-III-like peptide has specific follicle-stimulating hormone (FSH)-releasing activity and the authors propose that lGnRH-III or a closely related peptide is an FSH-releasing factor that does not release luteinizing hormone (LH). Although the existence of an FSH-releasing factor was proposed 35 years ago [21, 22], isolation, purification and sequencing of this molecule are still lacking.

In this framework the goal of the present work was to isolate, sequence and test the biological activity on FSH and LH release of the proposed GnRH that specifically releases FSH. Rat and hamster brain extracts were analyzed with HPLC and RIA to find the fraction that elutes in the same position with lamprey GnRH-III. The fraction was purified and sequenced, and tested for LH and FSH release.

Materials and Methods

Two sets of experiments were performed using two different batches of adult animals of both sexes. In the first experiment we used 50 hamsters *Mesocritus auratus* and 50 rats *Rattus norvegicus* (IBYME colony). The animals were killed by decapitation and the brain tissue was exposed. The hypothalami were removed and combined separately for each species (2.3 g from rats and 3.5 g from hamsters). The samples were immediately frozen on dry ice and stored at –70°C. Peptide extraction was done as previously described and followed by RP-HPLC [17]. Fractions containing immunoreactive (ir)-GnRH were identified with two polyclonal RIA systems. The remaining brain tissue from both species was analyzed in the same manner.

The second set of experiments was developed to purify and sequence the early-eluting ir-GnRH fractions previously identified. In this case, 731 rat brains (1,096 g) and 774 hamster brains (791 g) were used. Animals were quickly killed by decapitation. Brain tissue was exposed, removed, immediately frozen on dry ice and stored at –70°C until peptide extraction.

All procedures were performed according to protocols for animal use, as approved by the Institutional Animal Care and Use Committee (IBYME-CONICET) that follows NIH guidelines.

Peptide Extraction

Frozen pooled brains were powdered in a Waring blender with liquid nitrogen and peptide extraction was done as previously described [23]. Briefly, brain tissues were homogenized in acetone:1 *N* HCl (100:3, v/v) at 4°C and the extraction mixture was stirred at 4°C for 3 h. Insoluble material was filtered through Whatman No. 1 filter paper, reextracted in acetone:0.01*N* HCl (80:20, v/v) in 40% of the original volume, stirred for 5 min at 4°C and refiltered. The combined filtrates were extracted 5 times with petroleum ether (bp 30–60°C), as previously described [24]. Then brain extracts were reduced in volume in a vacuum centrifuge and filtered.

HPLC Purification

For prepurification in the second experiment, brain extracts were applied to a series of 10 SepPak cartridges (Waters, Milford, Mass., USA) and eluted with mobile phases A (0.5% trifluoroacetic acid, TFA) and B (0.5% TFA-80% acetonitrile, ACN). One-milliliter fractions were collected for 60 min and 5-μl aliquots from each fraction were subjected to RIA. ir-GnRH fractions were pooled, concentrated under vacuum and injected onto a reverse phase analytical column (Supelcosil LC-18). The sample was applied at the beginning of a 10-min isocratic period of 17% ACN in 0.25 *M* triethylammonium formate (TEAF; pH 6.5); then, ACN was increased to 24% over a 7-min period and held isocratically for 43 more minutes. One-milliliter fractions were collected and aliquots of 5 μl from each fraction were used for RIA. Early ir-GnRH fractions that eluted in the expected position of lGnRH-III were pooled and selected for further purification. Three successive RP-HPLC steps were performed with different reverse-phase HPLC columns and solvents (table 1).

Fractionation of the hypothalamic extracts in the first experiment was performed using only steps 1 and 2 from table 1. Each injection of the tissue extract was preceded by a blank run, which was assayed, to ensure that the column was not contaminated. After the tissue extracts, 300 ng of different standards were chromatographed under the same conditions on a different column for comparison.

Table 1. Steps of GnRH purification from hamster and rat brains

Step	Column			Mobile phase[1]		Sequential elution conditions		
	type	packing	size, cm	A	B	initial % B	final % B	time[2] min
1	Supelco	C18	0.45×25	0.25 *M* TEAF (pH 6.5)	100% ACN	17 17 24	17 24 24	10 7 43
2	Supelco	C18	0.45×25	0.13 *M* TEAP (pH 2.5)	100% ACN	17 17 24	17 24 24	10 7 43
3	Supelco	C18	0.45×25	0.05 *M* TFA (pH 2)	80% ACN/ 0.05% TFA	5 5 20 50 100	5 20 50 100 100	5 10 30 10 15
4	Vydac	Diphenyl	0.45×25	0.05 *M* TFA (pH 2)	80% ACN/ 0.05% TFA	5 5 20 50 100	5 20 50 100 100	5 10 30 10 15

Each line indicates sequential RP-HPLC system and elution program. TEAP = Triethylammonium phosphate.
[1] Balance of indicated mixture.
[2] Time to attain final percentage B from start of substep of HPLC program.

GnRH RIA Measurement

Fractions collected at each successive step in the RP-HPLC purification were assayed for ir-GnRH following established protocols [23]. RIAs were performed using polyclonal antisera GF-6 and PBL#49. Also, assays for the first experiment were performed using PBL#45 antiserum [11]. These antisera were chosen because of the high cross-reactivity against many GnRH variants. The RIA systems were as follows.

(1) GF-6 antiserum was raised by one of us (NMS) in rabbits against sGnRH. Used at a 1:25,000 final dilution with synthetic mGnRH as tracer and standard, cross-reactivities were mGnRH 100%, gpGnRH 106%, [Hyp^9]GnRH 79%, cGnRH-I 44%, cGnRH-II 3.9%, lGnRH-III 0.4%, sGnRH 69% [13]. (2) PBL#49, a generous donation of Dr. W. Vale (The Salk Institute), was used at a final dilution of 1:150,000 with sGnRH as tracer and standard giving the following cross-reactivities: mGnRH 93%, lGnRH-I 2%, cGnRH-I 333%, cGnRH-II 33%, sGnRH 100% [11]. (3) PBL#45, donated by Dr. W. Vale, was used at a final dilution of 1:250,000 with sGnRH as tracer and standard. PBL#45 showed the following cross-reactivities: mGnRH 100%, lGnRH-I 24%, cGnRH-I 133%, cGnRH-II 26%, sGnRH 100% [11].

Characterization of the Primary Structure

An aliquot of the peptide purified by RP-HPLC on a diphenyl column was injected into a microbore C18 column using 0.05% TFA and ACN for elution. Fractions were collected and analyzed with a Bruker Reflex time-of-flight instrument using an accelerating voltage of 31 kV and a reflectron voltage of 30 kV (100 MHz digitizer). The sample was applied to a thin layer of α-cyano-4-hydroxy cinnamic acid, allowed to dry and rinsed with water before analysis. Sequencing was initially attempted on 10% of each sample. Failure of this sequencing indicated a blocked N-terminus. Subsequent sequencing was carried out on the remaining material after digestion with pyroglutamyl aminopeptidase and microbore HPLC purification [25]. This was followed by RP-HPLC separation and sequence analysis by automated Edman degradation on a PE-ABI Procise 494 protein sequencer.

Synthetic GnRH Standards

lGnRH-III, [Hyp^9]GnRH, mGnRH and gpGnRH peptides were synthesized using a solid phase method on a methylbenzhydrylamine resin as previously described [26]. cGnRH-II and sGnRH were obtained from Peninsula Laboratories (Belmont, Calif., USA).

Bioactivity of GnRH Peptides

Synthetic lGnRH-III, [Hyp^9]GnRH, mGnRH and cGnRH-II peptides were tested for LH and FSH release in two rat anterior pituitary in vitro models.

Monolayer Cultures of Adenohypophyseal Cells

Anterior pituitary cells were obtained as previously described [27]. Briefly, anterior pituitaries from 12-day-old female rats were rapidly removed and placed in freshly prepared Krebs-Ringer bicarbonate buffer without Ca^{2+} and Mg^{2+}. Pituitaries were cut into small

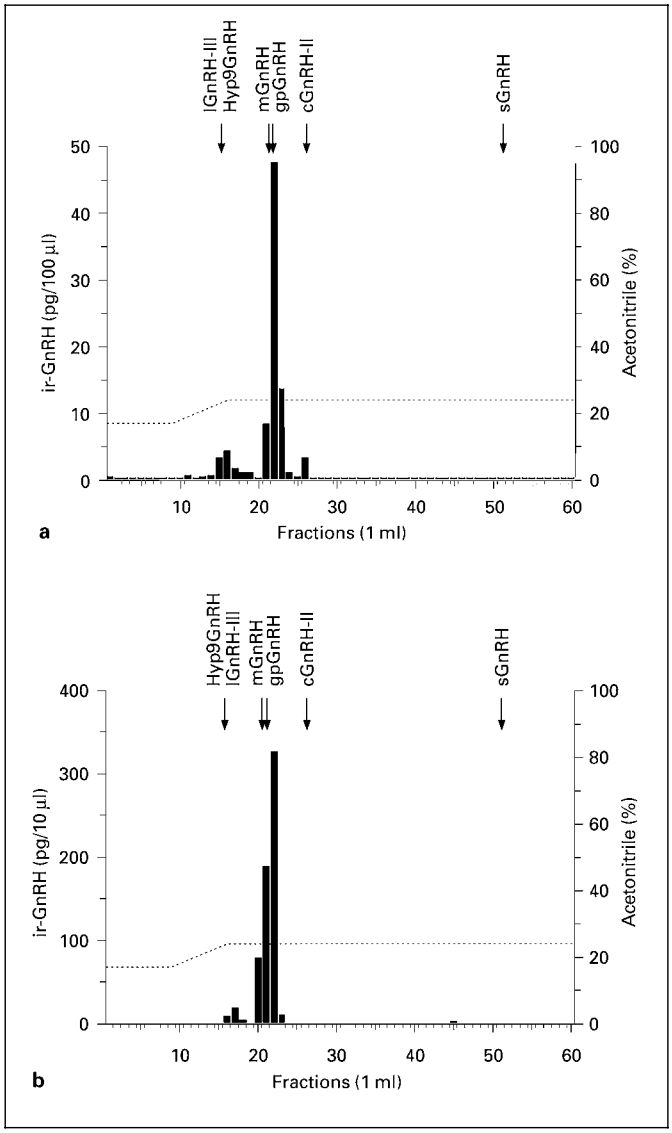


Fig. 1. RP-HPLC fractions from hamster's preoptic-hypothalamic area extract (**a**) and remaining brain (**b**) chromatographed with system 1 (TEAF, 0.25 *M,* pH 6.5) and analyzed with the PBL#49 RIA system. Arrows indicate the elution position of six different GnRH synthetic standards.

pieces and incubated in 0.2% trypsin for 30 min. After addition of DNase and limabean trypsin inhibitor, the fragments were dispersed into individual cells by gentle trituration and filtered through Nytex. Pituitary cells were plated in 96-well plates (50,000 cells/well) with Dulbecco's modified Eagle medium supplemented with 10% horse serum, 2.5% fetal calf serum, 1% MEM nonessential amino acids, nystatin and gentamicin (medium 1). After 4 days in culture, cells were washed twice with serum-free DMEM-F12 medium containing 2.2 g/l $NaHCO_3$ and 0.1% BSA (medium 2) and then incubated for 1 h with the different GnRH variants in a concentration range of 1×10^{-11} to 1×10^{-7} *M*. At the end of incubation, the medium was removed from each well and stored at –20°C until LH and FSH were analyzed by RIA after appropriate dilution with 0.01 *M* phosphate-buffered saline with 1% egg albumin. Cell cultures were repeated 4–5 times, and each time experimental groups consisted of 4 wells each. Results are expressed as ng/ml of medium from 50,000 cells and analyzed by repeated measures ANOVA.

Incubations of Hemipituitaries

Adult male rats were sacrificed by decapitation. The brains were exposed and anterior pituitaries removed, halved and preincubated in 0.75 ml Krebs-Ringer-CO_3HNa (5 m*M* ascorbic acid, pH 7.4) buffer (KRB) for 1 h at 37°C in an atmosphere of 95% O_2/5% CO_2 under agitation. Following this preincubation, hemipituitaries were incubated for 3 h in fresh KRB buffer without GnRH or containing graded concentrations (1×10^{-9} to 1×10^{-7} *M*) of mGnRH or lGnRH-III. Each hemipituitary was the control of the other half. Thereafter media were stored frozen at –20°C until LH and FSH RIAs were performed. Hemipituitaries were weighed. Hormonal values were expressed relative to the hemipituitary weight. Experiments were repeated 3 times. Results are expressed as ng/ml of medium/hemipituitary weight (mg) and analyzed by repeated measures ANOVA.

LH and FSH RIA Measurements

LH and FSH in samples of media were determined by RIA using kits provided by the NIDDK. Results are expressed in terms of RP3 rat LH and RP2 rat FSH standards. Assay sensitivities were 0.6 ng/ml for LH and 1.5 ng/ml for FSH. Intra- and interassay coefficients of variation for LH were 7.2 and 11.4% and for FSH 8.0 and 13.2%.

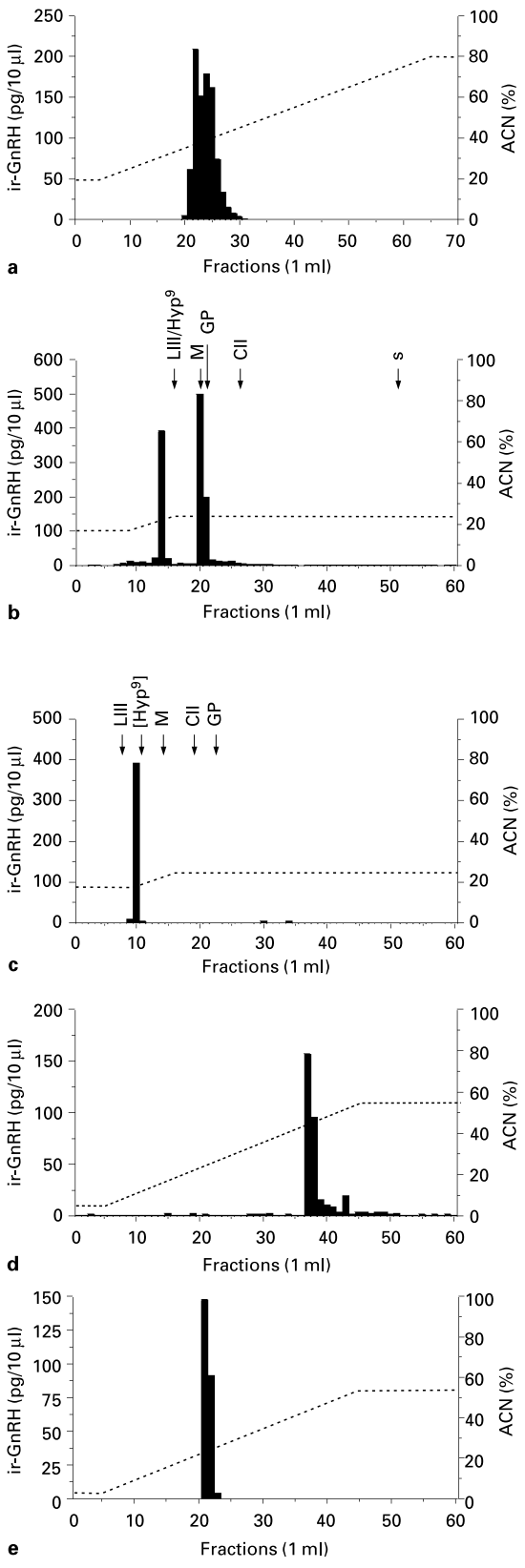
Results

To determine the elution position of different GnRH synthetic standards during purification, the first RP-HPLC system (table 1, step 1) was used. The retention times were 16 min for [Hyp^9]GnRH and lGnRH-III, 20 min for mGnRH, 21 min for gpGnRH, 26 min for cGnRH-II and 50 min for sGnRH.

GnRH Screening from Rat and Hamster Hypothalami

Extracts from hamster hypothalami, chromatographed with RP-HPLC step 1 (TEAF pH 6.5) and analyzed with PBL#49 RIA system, revealed three ir-GnRH peaks. The first one eluted in the same position as lGnRH-III/[Hyp^9]GnRH (fractions 15–16), the second peak showed the same elution position as mGnRH/gpGnRH (fractions 21–23) and the third one eluted at fraction 26 like the cGnRH-II synthetic standard (fig. 1a). The analysis of the remaining brain of the hamster using the same system revealed two immunoreactive peaks in fractions 16–17 and 20–22 (fig. 1b).

Additionally, we tested RP-HPLC fractions from rat hypothalami and the remaining brain, with the same



methodology, and two irGnRH fractions were revealed: fractions 16–17 and 20–22 (data not shown). PBL#45 antiserum produced similar results both in rats and hamsters (data not shown).

GnRH Purification

To isolate and sequence immunoreactive fractions that eluted in a similar position with lGnRH-III and [Hyp^9]GnRH, we purified more material using both hamster (fig. 2) and rat total brains (fig. 3).

Assays of GnRHs from hamster brain extract eluting from SepPak cartridges revealed one extended immunoreactive area between fractions 21–28 (fig. 2a). These fractions were pooled, concentrated and used for subsequent steps of purification.

The first RP-HPLC purification step revealed two ir-GnRH peaks (fig. 2b). The first one eluted between fraction 13 and 15 (peak I) near lGnRH-III/[Hyp^9]GnRH standards, and the second eluted between fractions 20–21 (peak II, mGnRH/gpGnRH elution positions). Early eluting fractions (peak I) were pooled and used for subsequent steps of purification. In the second RP-HPLC system using triethylammonium phosphate pH 2.5 as a mobile phase, a single ir-GnRH peak was observed in the same position to that of [Hyp^9]GnRH (fig. 2c). ir-GnRH fractions 9–11 from the previous step of purification were concentrated and reinjected using the third step of the purification. A major peak was observed in position 37–39 (fig. 2d) and was concentrated and subjected to the last step of purification. A single peak eluting from the diphenyl column in position 21–22 (fig. 2e) was used for microbore HPLC sequence analysis. A similar immunoreactive pattern was observed during the GnRH purification steps from rat brain tissue (fig. 3a, e).

Sequence and Mass Spectral Analysis

Initial attempts to analyze the peptide by chemical sequence analysis failed due to a blocked N-terminus for

Fig. 2. Elution of ir-GnRH fractions during chromatographic purification from hamster brains: prepurification: ACN/TFA (SepPak) (**a**), step 1: ACN/TEAF pH 6.5 (**b**), step 2: ACN/triethylammonium phosphate pH 2.5 (**c**), step 3: ACN/TFA pH 2.0 (**d**), step 4: ACN/TFA pH 2.0 (**e**). Immunoreactive fractions are indicated by bars. The final step with microbore HPLC is not shown. Dotted lines indicate ACN concentration of the mobile phase. Arrows indicate the elution position of the following standards: LIII = lGnRH-III; Hyp^9 = [Hyp^9]GnRH; M = mGnRH; GP = gpGnRH; CII = cGnRH-II; S = sGnRH.

both hamster and rat purified GnRH fractions. When the peptide was reanalyzed after treatment with pyroglutamyl aminopeptidase, the following sequence was obtained: His-Trp-Ser-Tyr-Gly-Leu-Arg-OHPro-Gly. The intact molecule mass of the purified peptide as determined by MALD mass spectrometry was 1,196.4 daltons (M-H+) for hamsters (fig. 4) and 1,196.3 daltons (M-H+) for rats (fig. 5), which are in good agreement with the theoretical mass of 1,196.57 daltons for the sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-OHPro-Gly-NH_2. The isolated native peptide was found to elute at the same percentage of ACN as the synthetic peptide with the above-mentioned sequence.

Bioactivity of Peptides: LH and FSH Release

The release of LH and FSH from 12-day-old female rat pituitary cells after the addition of synthetic forms of mGnRH, [Hyp^9]GnRH, lGnRH-III or cGnRH-II is shown in figure 6a. Similar to mGnRH ($p < 0.05$ at 10^{-9} and 10^{-7} *M*), both [Hyp^9]GnRH ($p < 0.05$ at 10^{-7} *M*) and cGnRH-II ($p < 0.05$ at 10^{-7} *M*) released LH from dispersed rat pituitary cells in vitro. However, lGnRH-III was not effective at any dose tested. It is important to note that although the cells were more sensitive to mGnRH than to [Hyp^9]GnRH or cGnRH-II with regard to LH release, the maximal LH response was not different when these three GnRHs were compared.

In the case of FSH-releasing activity, only mGnRH and cGnRH-II showed a significant response at 10^{-7} *M* concentration ($p < 0.05$), although dose-response profiles were apparent for both peptides (fig. 6b). [Hyp^9]GnRH did not evoke a statistically significant response but it showed a tendency to increase FSH release at the highest dose tested. In addition, lGnRH-III did not modify FSH release even at the highest concentration tested.

Our results, with regard to the gonadotropin-releasing effect of lGnRH-III in cultured cells, differed from those of other authors in another in vitro model. Therefore, we

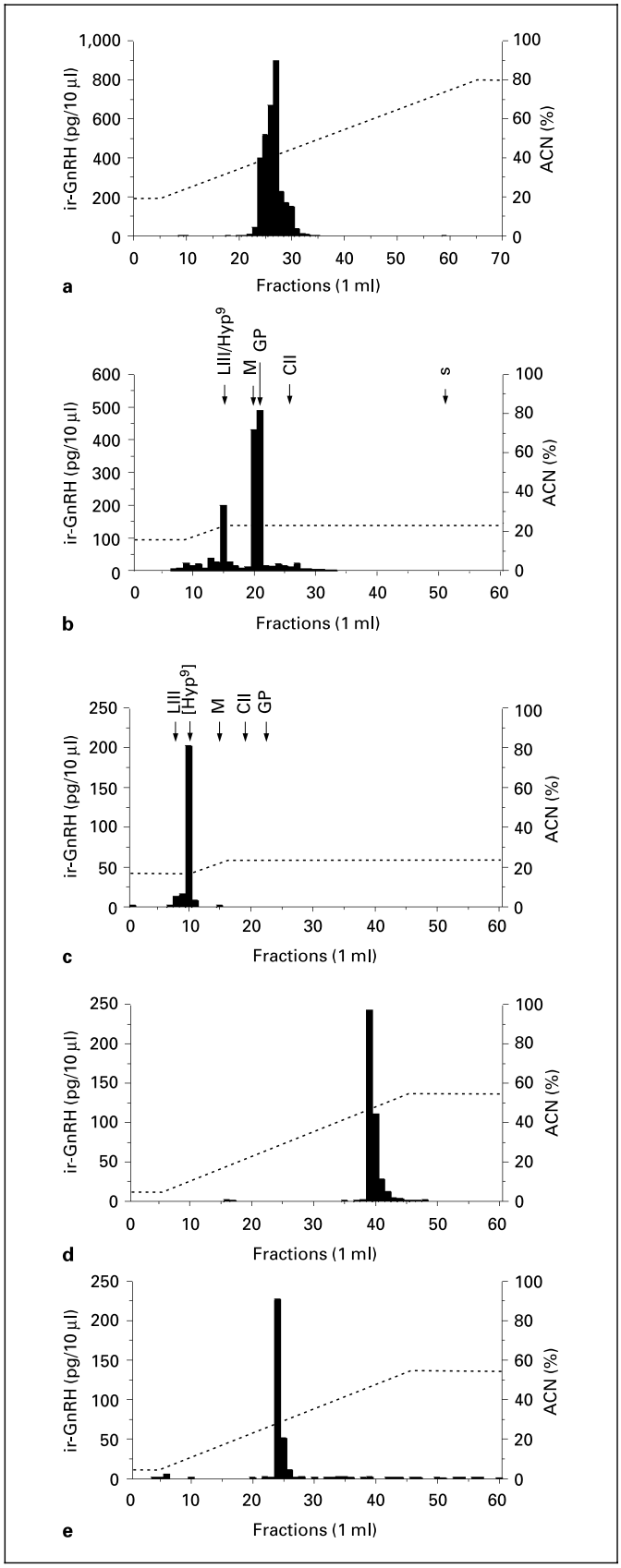
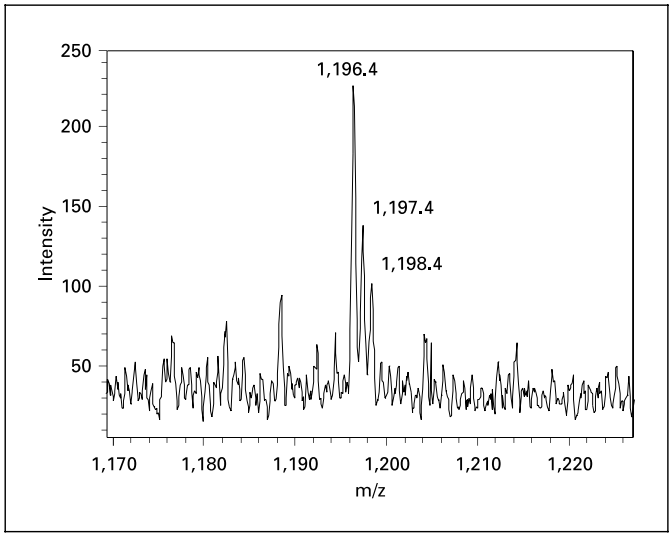


Fig. 3. Elution of ir-GnRH fractions during chromatographic purification from rat brains: prepurification: ACN/TFA (SepPak) (**a**), step 1: ACN/TEAF pH 6.5 (**b**), step 2: ACN/triethylammonium phosphate pH 2.5 (**c**), step 3: ACN/TFA pH 2.0 (**d**), step 4: ACN/TFA pH 2.0 (**e**). Immunoreactive fractions are indicated by bars. Final step with microbore HPLC is not shown. Dotted lines indicate ACN concentration of the mobile phase. LIII = lGnRH-III; Hyp^9 = [Hyp^9]GnRH; M = mGnRH; GP = gpGnRH; CII = cGnRH-II; S = sGnRH.



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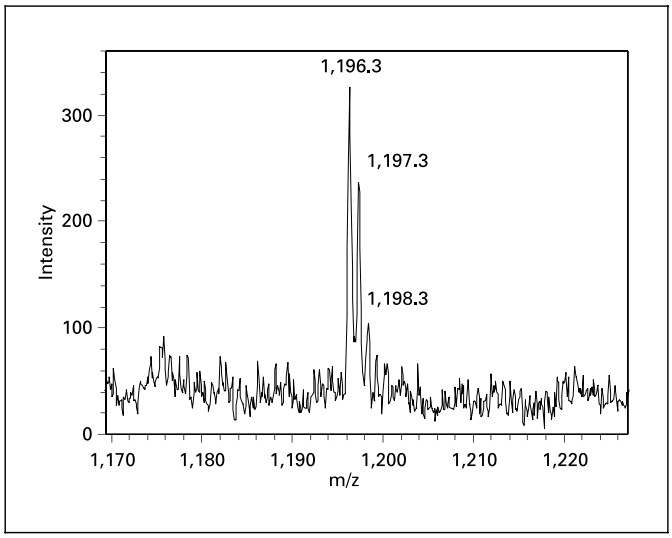

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Fig. 4. Negative mass spectrum of purified immunoreactive [Hyp^9]GnRH from hamster brain.

Fig. 5. Negative mass spectrum of purified immunoreactive [Hyp^9]GnRH from rat brain.

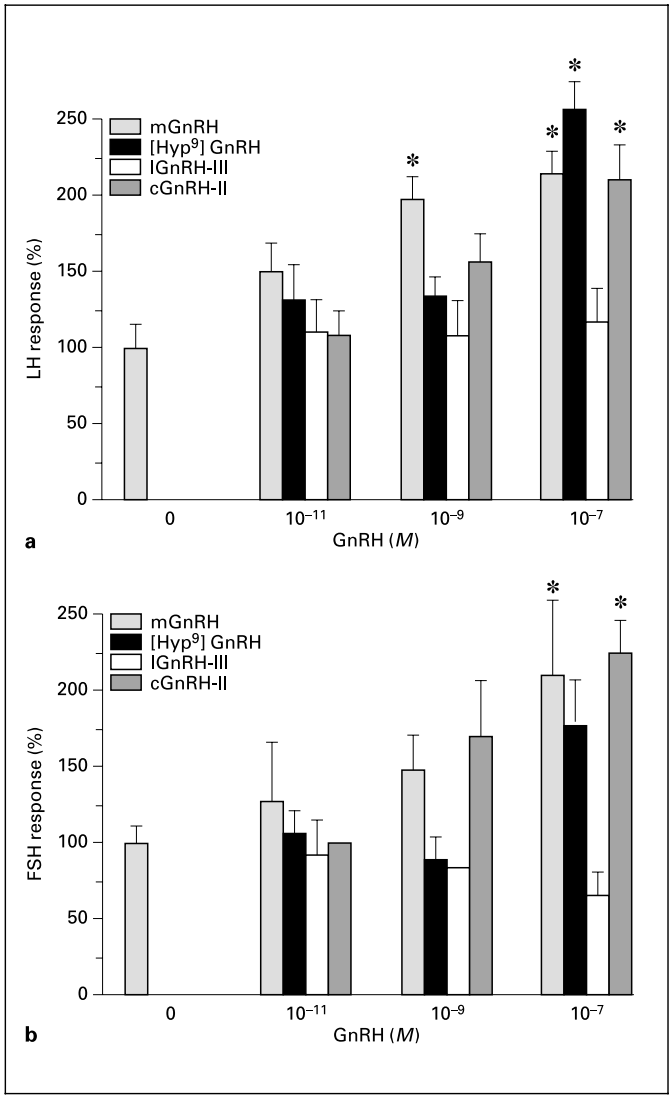

Fig. 6. Gonadotropin-releasing effect, LH (**a**) and FSH (**b**), of synthetic lGnRH-III, [Hyp^9]GnRH, mGnRH and cGnRH-II in pituitary cells of 12-day-old female rats incubated in vitro. The values are expressed as percentage of basal response (control). * $p < 0.05$ vs. control value (n = 5).

Table 2. Gonadotropin-releasing effect of lGnRH-III and mGnRH in male rat hemipituitaries incubated in vitro

	Control	mGnRH $1 \cdot 10^{-7}$ *M*	Control	lGnRH-III $1 \cdot 10^{-7}$ *M*
LH	15.82 ± 1.73	24.70 ± 3.90*	17.61 ± 1.84	15.70 ± 1.51
FSH	15.48 ± 1.61	25.20 ± 3.60*	18.61 ± 2.11	16.48 ± 1.68

* $p < 0.05$ vs. control value (n = 3).
The values are expressed as ng/ml medium/mg tissue.

tested the action of this peptide in male rat hemipituitary incubations in comparison to mGnRH. Whereas mGnRH induced a significant release of both LH and FSH, no effect was observed with lGnRH-III (table 2).

Discussion

The expression of two or more GnRH variants in the brain of a single species is well documented in different groups of vertebrates [28, 29]. In teleost fishes three different GnRH variants have been described in most of the species studied to date [2, 29]. Likewise, other nonmammalian vertebrates were shown to have multiple forms of GnRH some time ago. However, in mammals the expression of two or more forms of GnRH in a single species was demonstrated more recently. In addition to mGnRH, cGnRH-II has been shown to be present in species including rats, monkeys and humans [5, 13, 15, 30] and gpGnRH is reported for guinea pigs [16]. Further, different laboratories have used indirect techniques (not sequencing) to suggest that additional forms may be present in mammalian brains including unknown GnRH-like forms [11, 17, 18, 31] and lGnRH-like variants [19, 20, 32,]. In this context, it was proposed that lGnRH-III functioned as an FSH-releasing hormone [19, 20], which is a long-sought factor [21, 22].

The presence of multiple forms of GnRH in individual species has revealed that mammals share one GnRH form with all classes of vertebrates except the jawless fish. Thus, cGnRH-II has been found as a second form of GnRH in most vertebrates from jawed fish to placental mammals; cGnRH-II has been isolated and its primary structure determined from chicken, alligator, frog, seabream, tilapia, pacú, catfish, herring, pejerrey, dogfish and ratfish [2, 29]. Also, the sequence of cDNA encoding cGnRH-II was reported in several fish [33, 34] and mammals [7, 14, 15]. Cells expressing cGnRH-II are magnocellular and mainly located in the caudal diencephalon and the anterior midbrain tegmentum, which together are known as the posterior or midbrain GnRH system [35].

In this study, the pilot experiment on hamster and rat hypothalamic tissue extracts showed at least two irGnRH peaks; one peak was coincident with the elution position of lGnRH-III/[Hyp^9]GnRH synthetic standards and the other peak eluted in the position of mGnRH/gpGnRH standards. Also, a third peak was detected in the case of the hamster hypothalamic extract eluting in the same position as cGnRH-II synthetic standard. A late-eluting peak containing sGnRH was not observed. The lack of sGnRH is in contrast with the results from other authors, who postulated the presence of this variant in rat brain by immunohistochemical methods [31].

Our hypothalamic data suggest that three different GnRH peptides are found in the hypothalamic area, and suggest that all three forms could be related, directly or indirectly, to the control of pituitary hormone secretion. GnRH peptides are present in both neuronal cell bodies and axons. Therefore, peptides within the hypothalamus could be in axons that are passing through the hypothalamus or that terminate in areas other than the median eminence. Traditionally, cGnRH-II cell bodies were considered to be located mainly in the midbrain, although there is a recent report that cGnRH-II can be synthesized within the hypothalamus [15]. In hamster brain we detected cGnRH-II immunoreactivity in hypothalamic extracts, but not in the remaining brain. One possible explanation is that the cGnRH-II was stored in the axon terminals that end within the dissected region.

Our data show another HPLC peak that coelutes with both lGnRH-III and [Hyp^9]GnRH. Other reports have presented immunological and chromatographic evidence in the presence of lGnRH-III in sheep and rat brains [19, 20]. In addition, the same authors proposed that lGnRH-III is the long-sought FSH-RH because the peptide had an FSH-releasing activity with little effect on LH. In this framework it was important to purify the early eluting ir-GnRH molecule to identify the structure and to test its biological action on LH and FSH secretion.

Our results demonstrate that the early-eluting irGnRH molecule is [Hyp^9]GnRH, which has been previously described [4]. Its actions on FSH and LH secretion were as originally demonstrated [36], but certain points need to be emphasized. This is the first demonstration of the presence of [Hyp^9]GnRH by a primary sequence using Edman degradation in mammals. When [Hyp^9]GnRH was first discovered, it was very elegantly elucidated by indirect methods: mass spectrometry, amino acid composition and coelution experiments [4]. As originally described, [Hyp^9]GnRH is expressed in the hypothalami of man, rat, sheep and frog [4]. In addition, our sequence data on hamster and rat, and previously reported chromatographic and immunological data on rhesus monkey [13], guinea pig [37] and Mexican leaf frog [38] brain extracts support the idea that expression of [Hyp^9]GnRH is a common feature in mammals and amphibians. This fact also emphasizes the possibility of posttranslational modification by hydroxylation of Pro^9 in the other 12 GnRH variants or modification of other residues in any of the GnRH family members.

To date, the physiological actions of [Hyp^9]GnRH are not well known. It is reported that [Hyp^9]GnRH is released from the hypothalamus by different mechanisms in adult [39] and developing rats [40] and can potentially act on the secretion of human chorionic gonadotropin secretion from perifused placental cells [41]. Nevertheless, its actions are not fully understood. It is also important to note that, although the presence of [Hyp^9]GnRH was demonstrated in the hypothalamus, olfactory bulb and hippocampus in the rat [42], it is not known whether [Hyp^9]GnRH is in the same or different neurons compared to mGnRH.

Further, the presence of lGnRH-III was not detected in either rat or hamster brains. If this molecule were expressed in any of these animals, its presence should have been revealed as an additional early peak when the ir-GnRH was chromatographed in the RP-HPLC system 2 (table 1, step 2). In terms of evolution, this is not surprising. To date, lGnRH-III expression has not been demonstrated conclusively in any vertebrate except lampreys [43]. Indirect data suggested its presence in an early-evolved teleost fish, the white sucker [44] and its expression was also postulated in mammals [19, 20].

With respect to the biological activity on LH and FSH secretion, we have used 12-day-old female rats because it has been demonstrated that this is the most sensitive period to GnRH [45] and LH and FSH levels reported in this work are in line with those of others in this respect [46]. cGnRH-II appeared to have a similar potency to that of mGnRH on both FSH and LH secretion in the system used here. With regard to LH, the sensitivity to mGnRH was higher than to cGnRH-II because it elicited a significant response at a concentration 100 times lower. The similar potency of mGnRH and cGnRH-II is in contrast to that observed in other systems [28, 47], but is in agreement with the high binding affinity of cGnRH-II for the GnRH receptor in humans [48]. Millar et al. [49] have shown that a cGnRH-II receptor is expressed in gonadotropes and appears to modulate the type I (mGnRH) receptor and the secretion of LH and FSH. Of the other two synthetic peptides tested, [Hyp^9]GnRH actions on FSH and LH secretion followed the originally described pattern [36], but lGnRH-III had no action on LH or FSH secretion. Furthermore, though not statistically significant, lGnRH-III tended to inhibit basal FSH secretion. These data agree with results recently presented by Lux-Lantos et al. [50] and Kovács et al. [51] that lGnRH-III is not the mammalian FSH-RH. Kovács et al. [51] presented evidence showing that lGnRH-III is a weak agonist for the mGnRH receptor with a relatively stronger but not a selective FSH-releasing effect in vivo. However, in vitro they found that lGnRH-III stimulated LH and FSH secretion only at doses 500–1,000 times higher than those required for mGnRH. In rat pituitary cells, no selective FSH-releasing activity by lGnRH-III was observed.

Our results in mammals showed that lGnRH-III is not expressed and that the synthetic form of lGnRH-III does not act as an FSH-releasing hormone, making it highly improbable that lGnRH-III is the postulated FSH-RH. Moreover, this is the first study demonstrating the presence of [Hyp^9]GnRH in two mammalian brains by primary sequence.

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